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2700197 ESI2567-1 102776
P01/7700 25.00 - 9722481.0

2. Patent application number 25 OCT 1997
(The Patent Office will fill in this part) **9722481.0**

3. Full name, address and postcode of the or of each applicant (underline all surnames)
The Secretary of State for Defence
Defence Evaluation & Research Agency
Ively Road, Farnborough, Hampshire, GU14 0LX

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

GB

54510004

4. Title of the invention
Expression System

5. Name of your agent (if you have one)

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Description 8

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Abstract 1

Drawing(s) -

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11. I / We request the grant of a patent on the basis of this application.

Signature *S R Skelton*
S R Skelton

Date 23/10/97

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DUPLICATE

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Expression System

5 The present invention relates to a method of obtaining polypeptides or protein products such as enzymes and in particular luciferases, which are substantially free of undesired contaminants such as other enzymes for instance adenylate kinase, usually found in expression products, to recombinant host cells, vectors and nucleotide sequences useful in these methods.

10 Recombinant DNA technology has allowed the production of a vast range of useful protein and polypeptide products efficiently. Essentially, host cells which may be eukaryotic or prokaryotic, are engineered so that they express the desired products. Culture of the recombinant cells results in the production of the desired products, sometimes in large quantities. Particularly useful host cells are prokaryotic cells such as bacteria like *E. coli* or *Bacillus spp.*. These cells multiply rapidly and so culture of the cells means that large quantities of the desired products may be
15 obtained either by lysis of the cells, or by extraction from culture supernatant if the cells can be induced to secrete these products. The desired product must then be extracted from the culture medium and purified so that it is free of all the other proteins present as a result of the cell culture process.

20 Purification can sometimes be difficult to achieve as the culture medium contains a large amount of other proteins and products, some of which may have similar properties such as size, charge or affinity for a particular substrate to the desired protein, making complete purification using for example chromatographic techniques difficult. For many applications, small quantities of contaminant proteins may not present a significant problem in terms of the end use of the product.
25 For other applications however, the presence of even minute quantities of particular undesired products may be extremely damaging with regard to the utility of the product. A particular case may relate to enzymes found in host cells. If the activity of such molecules impedes or is contrary to that of the desired product, even tiny amounts present as a contaminant may hinder or obstruct the end use of the product.

Where the damaging contaminant is the product of a non-essential gene of the host cell, the problem may be addressed by inactivating that gene, for example by deletion, mutation or even by using anti-sense RNA constructs to "switch off" the gene. However, where the gene product is essential for the survival of the host cell such an approach is not possible as clearly, the host cell would not survive and so the production process would cease. The same may apply where inactivation reduces the efficiency of the cell by a significant amount so that the production process is rendered non-viable.

For example, luciferase enzyme is well known as a labelling tool in biological research. It is a useful enzyme in that when combined with luciferin and ATP, it provides a useful signalling system, providing a fluorescent signal which can be read easily using for example luminometer devices. Luciferase can be isolated from natural sources such as fireflies and some beetles. For large scale production however, luciferase is generally produced by expression from a prokaryotic host, such as *E. coli*.

One particular application to which luciferase may be put is in an assay for detection of cellular components such as ATP or enzymes such as adenylate kinase (AK), as described in European Patent Application No. 94904295.6. Such assays are useful in detecting the presence of microorganisms in a particular environment. For these purposes, the presence of cellular components which are the target of the assay in the luciferase reagent will produce levels of "background" noise which will have to be taken account of when interpreting results obtained using these products. This is a particular problem in the adenylate kinase assay, which has a high level of sensitivity.

Adenylate kinase is the enzyme which catalyses the reaction which converts ADP to ATP, the essential energy component of the cell. In the assay mentioned above, the amount of AK is measured by adding ADP to the reagent mixture which is converted to ATP by any AK present.

The ATP product is then detected using luciferase/luciferin system. The use of AK as the substrate in the assay produces an amplification of the signal many times over. This is very useful when very small quantities of cellular content, for instance, very small numbers of microorganisms are present in the sample.

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However, equally, any residual AK in the luciferase reagent used in the assay will generate a similarly amplified signal and may generate false positive results. For this reason, highly stringent purification of the luciferase reagents used in this assay has been necessary. Inactivation of the AK gene in the expression host used to produce the luciferase in the first place is not an option as AK is an essential enzyme which allows the cell to function. Without this enzyme, the host cell would die.

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The applicants have devised a new technique where the problem of contamination of products of recombinant DNA technology by undesired or even harmful products can be minimised.

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In particular, the invention provides a method for producing a polypeptide product which is substantially free of an undesired protein, the process comprising culturing a host cell which is able to express said polypeptide product and which is able to express said undesired protein only in a mutant form which form has the activity of the corresponding native protein under culture conditions but is unstable under conditions at which the said polypeptide product remains stable; and recovering the desired product, wherein either the host cell culture or the recovered product is subjected for a sufficient period of time to the conditions under which the undesired protein is unstable so as to denature the undesired protein.

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In this manner, the undesired protein, which is for example, an essential protein for the host cell, is produced during the culture process so that the host cell can continue to multiply. Once the production is complete, a suitable batch of the culture medium is subjected to conditions under which the undesired protein is denatured. Further multiplication of the host cells is not required

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at this point and so the death of the cells is immaterial. The desired product may then be recovered from the culture residue.

5 Alternatively, the desired product may be recovered from the culture medium and then subjected to the conditions under which the undesired protein is denatured, so that any contaminant protein will not adversely affect the product activity.

As used herein the term "polypeptide product" refers to any polypeptide which may be expressed from a host cell, including large polypeptides and proteins.

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The sorts of conditions which may be used to bring about a denaturation of the undesired protein may be conditions of temperature, pH or the presence of particular reagents which bring about denaturation. For example, the mutant protein may be made to be either thermolabile or acid-labile at temperatures or pH levels at which the desired product remains intact. Other possibilities
15 include making the undesired protein unstable in the presence of chaotropic agents such as urea or detergents, or oxidising agents or proteolytic agents.

Suitable mutant forms of the protein may be identified by known techniques. For example, random mutagenesis can be used to produce a range of mutant genes. These may be cloned into
20 expression hosts such as *E. coli* using conventional technology and the resultant clones screened for the desired instability. Once identified, the clones which are, for example, thermolabile, are sequenced and the mutation which results in this property are identified. More than one mutation may result in the labile nature required, and it may be preferable to form mutants with two or more such mutations as this may increase the observed effect.

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For example, the gene which encodes adenylate kinase of *E. coli* is known (Brune, Schumann and Wittinghofer, Nucleic Acids Research, (1985) 13, No. 19, 7139-7150; P. Liang et al., Gene (1989) 80, 21-28).

In a particular embodiment of the invention, colonies of *E. coli* are mutated by non-specific methods and the mutants differentially screened at 25°C and 37°C. Mutants containing a thermolabile adenylate kinase gene should only be able to grow at 25°C and not at 37°C. These can then be screened for AK activity at various temperatures to select out AK mutants.

It has been found that mutation at position 87 in that sequence and/or position 107 in the sequence, produces a mutant form of adenylate kinase enzyme which is labile at low temperatures. Therefore, an alternative approach is to clone the adenylate kinase gene into a suitable vector such as Promega plasmid "pALTER-1". Site-directed mutagenesis of the amino acids at positions 87 and 107 for example using PCR based methods will give a gene product which has altered thermolability. Screening of these mutants as described above will indicate which substituent amino acids at these positions give adenylate kinase which is more thermolabile than the native protein.

The construct thus obtained can then be used to transform a competent host strain of *E. coli* that is amenable to recombination such as JM83, in order to allow recombination of the mutant gene into the host chromosome, preferably in place of the existing adenylate kinase gene. Successful recombinants will only contain the mutant adenylate kinase gene encoding a thermolabile product. Adenylate kinase is an essential enzyme to the cell; therefore mutants will only be able to survive at a lower permissive temperature than wild type or standard laboratory strains.

Conversely and additionally, the desired polypeptide product may be engineered so that its tolerance to the conditions under which the undesired protein is denatured is increased. For instance, in the case of luciferase enzyme, several thermostable mutants are known in the art and these may be employed in the method of the invention. Alternatively other thermostable mutants or mutants which have increased acid stability etc. can be prepared using similar techniques. In this case, the screening process will select those mutants which have increased tolerance rather than decreased tolerance to the condition being used to denature the undesired polypeptide.

Constructs of the type described above as well as host cells transformed with said constructs and methods of producing them form further aspects of the invention.

- 5 Thus the invention further provides a recombinant cell which comprises a first nucleotide sequence which encodes a desired polypeptide such as a luciferase, under the control of regulatory elements which allow expression of said polypeptide, and wherein a gene which encodes a protein which is undesirable as a contaminant in preparations of said polypeptide product such as adenylate kinase, is mutated such that the protein expressed is unstable under conditions in which the polypeptide
10 product remains stable.

Preferably the luciferase is a thermostable luciferase, whilst the adenylate kinase is a thermolabile mutant.

- 15 These recombinant cells may contain one or more selection markers which are used in the production process.

The recombinant cell may be prokaryotic or eukaryotic but is preferably a prokaryotic cell such as a recombinant *E. coli* cell

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- The invention further provides a method for producing a recombinant cell according to any one of claims 10 to 14 which method comprises in any order (a) transforming a host cell with a vector which encodes said undesired protein in a form which is unstable under given conditions, subjecting transformants to said conditions and detecting those in which protein product is
25 denatured, and (b) transforming said host cell with a vector which encodes a desired polypeptide which is stable under said conditions and a first selection marker, and using the first selection marker to detect stable transformants

The vector which encodes said undesired protein in a form which is unstable under given conditions may further comprise a selection marker which is different to said first selection marker, and stable transformants are selected.

- 5 Suitable selection markers comprise particular different antibiotic resistance genes.

The invention will now be particularly described by way of example.

Example 1

- 10 PCR primers can be designed as is conventional in the art with a view to amplifying a gene encoding an undesired protein such as adenylate kinase. In this case, AK1 and AKR1013 from Cruachem may be used either as provided or optimised for instance using the Perkin Elmer 2400, and then using the Sigma PCR optimisation kit.
- 15 The PCR product which will be the wild-type adenylate kinase gene or alternatively a mutant adenylate kinase gene already known to produce thermolabile adenylate kinase such as strain CV2 is then cloned into a suitable vector, such as the pALTER-1 from Promega, a plasmid based on pBR322. This has disabled antibiotic resistance genes to facilitate mutagenesis. [CV2 is known (Proc. Natl. Acad. Sci. USA, (1970) 65:737) and may be obtained from the *E. coli* Genetic Stock
- 20 Centre, 355 Osborn Memorial Laboratories, Box 208104, Yale University, New Haven, CT06520-8104, USA.]
- In one embodiment, two adenylate kinase genes can be inserted in tandem separated by an antibiotic resistance gene. The elements of the construct may each require individual promoter
- 25 sequences, but increased expression would result.

Mutations can then be introduced, for example at amino acid positions 87 where proline may be changed to serine for instance (which involves only one base change) and/or at amino acid position 107 where leucine may be changed for instance to glutamine. The preferred codon usage for

adenylate kinase in *E. coli* is known. Thus all possible amino acids can be substituted at these positions, and the most thermolabile enzyme which still retains enough activity to allow cell survival will be selected. Preferably, the construct further contains a selection marker gene such as an antibiotic resistance marker downstream of the adenylate kinase gene so that screening for positive recombinants may be effected easily. The selection marker will have to be different to the one used later in the procedure in order to select positive transformants as described below.

Once a suitable thermolabile adenylate kinase producing mutant has been found, the mutated plasmid can be used to transform a recombinant positive strain of *E. coli* such as JM83 as is known in the art. The recombinants will then be screened for example by differential screening at 37°C and a much lower temperature, for example 20°C. Positive recombinants may be identified using the selection marker, for example antibiotic resistance where such a marker is present.

Colony blots can then be performed with the resultant mutant oligo to confirm that the mutant sequences are the desired ones. This will be done using chemiluminescent (e.g., HRP) labelled probes. Sequencing can also be carried out in addition to or as well as colony blots.

The *E. coli* host produced in this way has a chromosomal mutation in its adenylate kinase gene that causes the adenylate kinase produced to be temperature sensitive. A luciferase plasmid, preferably a thermostable luciferase plasmid, for example as described in European Patent Application No. 92110808.0 or WO95/25798, can then be introduced to the host such that the luciferase can be produced at a temperature that is permissive to the adenylate kinase. The culture can be raised to a higher temperature to denature the adenylate kinase which is present.

Purification of the thermostable luciferase enzyme can then be carried out using the standard methods devised previously for example as disclosed in WO95/25798.

Claims

1. A method for producing a polypeptide product which is substantially free of an undesired protein, the process comprising culturing a host cell which is able to express said polypeptide product and which is able to express said undesired protein only in a mutant form which form has the activity of the corresponding native protein under culture conditions but is unstable under conditions at which the said polypeptide product remains stable; and recovering the desired product, wherein either the host cell culture or the recovered product is subjected for a sufficient period of time to conditions under which the undesired protein is unstable so as to denature the undesired protein.
2. A method according to claim 1 wherein the host cells are cultured for a period which is sufficient to allow production of polypeptide product, and then a batch of said culture is subjected to the said conditions under which the undesired protein is unstable for a sufficient period of time to denature the undesired protein, and the polypeptide product is recovered.
3. A method according to claim 1 or claim 2 wherein the conditions at which the undesired protein is denatured and the polypeptide product remains intact are temperature conditions
4. A method according to claim 3 wherein said temperature conditions are elevated temperatures.
5. A method according to claim 4 wherein the elevated temperature is 37°C or more.
6. A method according to claim 1 or claim 2 wherein the conditions at which the undesired protein is denatured and the polypeptide product remains intact are pH conditions.

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7. A method according to any one of the preceding claims wherein the desired polypeptide product is luciferase and the undesired protein is adenylate kinase.
8. A method according to claim 7 wherein the adenylate kinase is thermolabile at a temperature of 37°C or more.
9. A method according to claim 7 or claim 8 wherein the adenylate kinase includes mutations at amino acids 87 or 107 in the sequence of the *E. coli* adenylate kinase.
10. A recombinant cell which comprises a first nucleotide sequence which encodes a desired polypeptide under the control of regulatory elements which allow expression of said polypeptide, and wherein a gene which encodes a protein which is undesirable as a contaminant in preparations of said polypeptide product is mutated such that the protein expressed is unstable under conditions in which the polypeptide product remains stable.
11. A recombinant cell according to claim 10 wherein the said desired polypeptide comprises a luciferase and the said undesired protein comprises adenylate kinase.
12. A recombinant cell according to claim 10 of claim 11 which further comprises at least one selection marker.
13. A recombinant cell according to any one of claims 10 to 12 which comprises a prokaryotic cell.
14. A recombinant cell according to claim 13 which comprises a recombinant *E. coli* cell.
15. A method for producing a recombinant cell according to any one of claims 10 to 14 which method comprises in any order (a) transforming a host cell with a vector which encodes said

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Desired protein in a form which is unstable under given conditions, subjecting transformants to said conditions and detecting those in which protein product is denatured, and (b) transforming said host cell with a vector which encodes a desired polypeptide which is stable under said conditions and a first selection marker, and using the first selection marker to detect stable transformants.

16. A method according to claim 15 wherein the vector which encodes said undesired protein in a form which is unstable under given conditions further comprises a selection marker which is different to said first selection marker, and stable transformants are selected.

17. A method according to claim 15 or claim 16 wherein said selection markers comprise particular different antibiotic resistance genes.

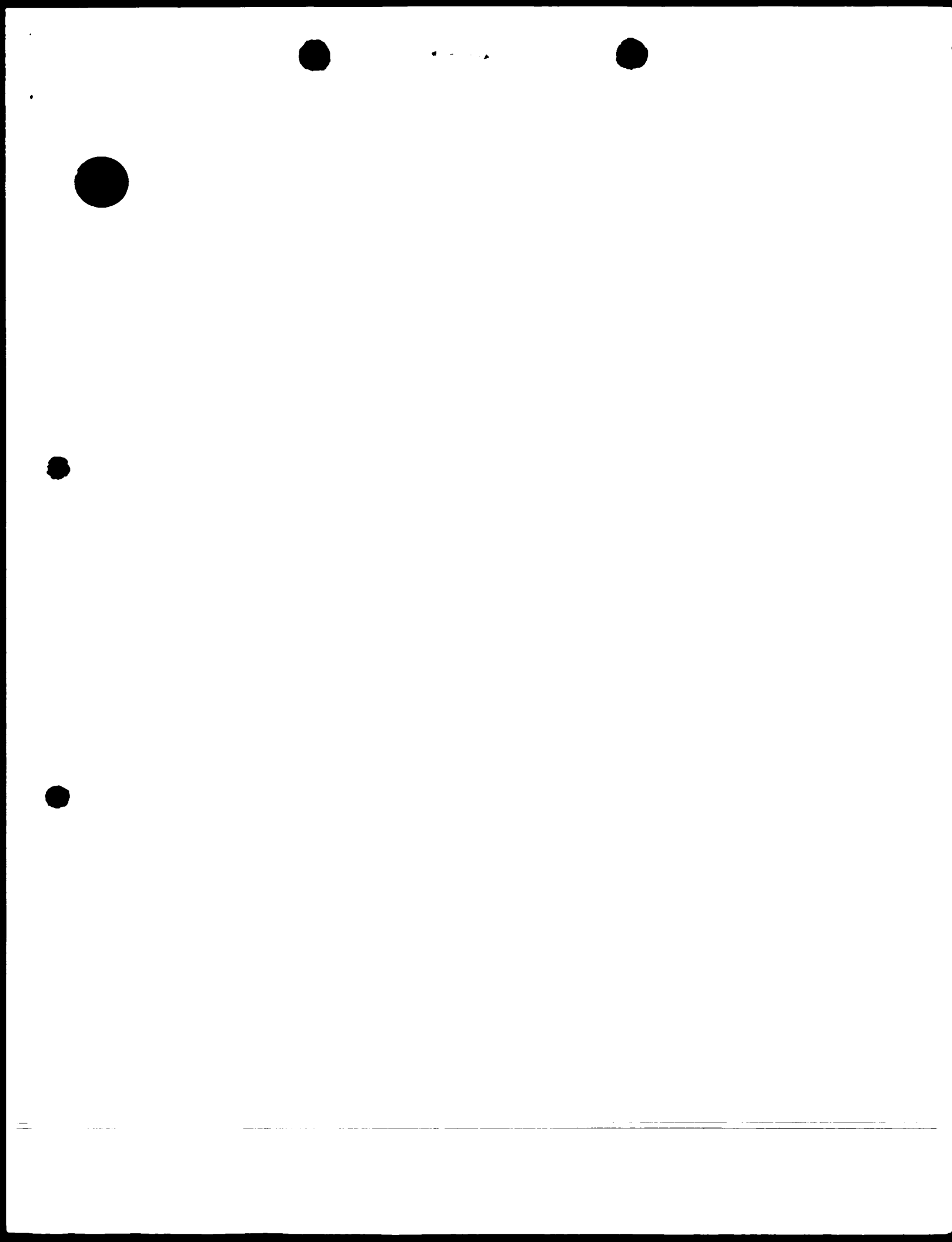
18. A polypeptide product which is substantially free of an undesired protein, as obtained by a method according to any one of claims 1 to 9.

12

Abstract

A method for producing a polypeptide product which is substantially free of an undesired protein, the process comprising culturing a host cell which is able to express said polypeptide product and which is able to express said undesired protein only in a mutant form which form has the activity of the corresponding native protein under culture conditions but is unstable under conditions at which the said polypeptide product remains stable; and recovering the desired product, wherein either the host cell culture or the recovered product is subjected for a sufficient period of time to conditions under which the undesired protein is unstable so as to denature the undesired protein.

The method allows the production of products which are substantially free of particular undesired contaminants.



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